

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
18 November 2004 (18.11.2004)

PCT

(10) International Publication Number  
**WO 2004/099256 A1**

(51) International Patent Classification<sup>7</sup>: **C08B 37/00**,  
37/10

(21) International Application Number:  
PCT/EP2004/050723

(22) International Filing Date: 6 May 2004 (06.05.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
03076388.2 9 May 2003 (09.05.2003) EP

(71) Applicant (for all designated States except US): LABO-  
RATORI DERIVATI ORGANICI S.P.A. [IT/IT]; Via M.  
Barozzi, 4, I-20122 Milano (IT).

(72) Inventors; and

(75) Inventors/Applicants (for US only): DE AMBROSI,  
Luigi [IT/IT]; Via G. Carducci, 8, I-13048 Santhià (VC)  
(IT). BENSI, Donata [IT/IT]; Via XX Settembre, 18,  
I-13100 Vercelli (IT). VISMARA, Elena [IT/IT]; Via G.  
Colombo, 81/A, I-20133 Milano (IT).

(74) Agent: SERRAVALLE, Marco; Via Benvenuto Cellini,  
11, I-20090 Segrate(MI) (IT).

(81) Designated States (unless otherwise indicated, for every  
kind of national protection available): AE, AG, AL, AM,  
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,  
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,  
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,  
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,  
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,  
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,  
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,  
ZW.

(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,  
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,  
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI,  
SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,  
GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**

— of inventorship (Rule 4.17(iv)) for US only

**Published:**

— with international search report

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PHYSICAL DEPOLYMERIZATION OF GLYCOSAMINOGLYCANS AND PRODUCTS OB-  
TAINED THEREFROM

(57) Abstract: The invention relates to a process for the depolymerization of glycosaminoglycans characterized by the use of  
UVC radiation. The invention also relates to the intermediate depolymerized heparin obtained by the process. The intermediate  
depolymerized heparin can be dissolved in a buffer solution and fractionated by Gel Permeation for obtaining the desired Molecular  
Weight.

WO 2004/099256 A1

**Process for the physical depolymerization of glycosaminoglycans and products obtained therefrom.**

**State of the art**

5 Glycosaminoglycans are natural products of large pharmaceutical interest. Among the most widely used we can mention heparin, dermatan, heparansulphate and chondroitines.

The molecular weight of the natural products varies considerably and normally ranges from 5 to 40 kDa. It is however known that, for certain applications, lower molecular  
10 weights lead to higher pharmacological activity. The high molecular weight of these compounds often renders impossible their oral administration. Furthermore, it is known that specific activities of glycosaminoglycans are related to relatively short saccharide sequences. Thus, it would be very advantageous to depolymerize glycosaminoglycans reducing the molecular weight without losing the active sites present in the molecule.

15 The chemical depolymerization of glycosaminoglycans is well known in the art and leads to glycosaminoglycans of lower MW but also with a lower S content.

EP 394 971 discloses an enzymatic or chemical depolymerization process. The obtained heparin oligomers have a sulphur content lower than 9%.

WO 90/04607 discloses a depolymerization of heparin and dermatansulfate by the use  
20 of  $\text{H}_2\text{O}_2$  and  $\text{Cu}^{2+}$ . The ratio  $\text{SO}_3^-/\text{COO}^-$  is slightly lower than in the starting heparin.

US 4,987,222 discloses a method for the depolymerization of heparin by the use of gamma rays. The examples disclose the preparation of heparin of Mw around 5,000 Da and with a high S content. However, the heparin produced by this method presents a certain amount of degradation products as a result of uncontrolled side reactions.

25 It is therefore desirable to reduce the molecular weight of glycosaminoglycans without substantially modifying the chemical structure of the same.

It is known in the art that the irradiation of glycosaminoglycans with UV leads to their degradation. For example, in Polymer Photochemistry 6 (1985) 465-474, Khan et al. studied the effect of UV radiation on aqueous solution of heparin-Ca salts.

30 Depolymerization was detected, but it was only considered as a degradation process, not as a potential industrial process for the controlled depolymerization of heparin.

**Summary of the invention**

The present invention relates to a physical process for the depolymerization of glycosaminoglycans by the use of UVC radiation having a peak in the range from 245 nm to 260 nm. It also relates to the glycosaminoglycans obtained by this process.

5

**Detailed description of the invention**

The present invention provides a physical depolymerization process which reduces the molecular weight of glycosaminoglycans without substantially modifying the chemical structure of the same.

- 10 The objective is achieved through use of UVC radiation. When using heparin as a starting material, this process results in a low to ultra-low molecular weight heparin characterized by high S content.

The starting materials to be used in the process according to the present invention are natural glycosaminoglycans such as heparin, heparansulphate, dermatane and  
15 chondroitine. Other suitable starting materials are derivatives of glycosaminoglycans obtained by known methods. Thus, for example, the N-acetyl or N-sulphate groups of the residues of hexosamine can be transformed in amino groups through N-desulphation or N-deacetylation reactions and the sulphate groups of the uronic acids through desulphation reactions can give rise to epoxy groups.

- 20 In another embodiment, it is possible to use as a starting material for the process of the present invention a glycosaminoglycane which has already undergone a depolymerization process either chemical, physical or enzymatic. Examples of physical depolymerization methods that can be used in combination with the process of the invention are described in WO 03/076474 (LDO) and WO 2004/000886 (LDO), which  
25 are herewith incorporated by reference. The use of partly depolymerized glycosaminoglycans is for example relevant in case of heparin which has undergone an acid pretreatment that has as a side effect partial depolymerization, or when depolymerizing functionalized glycosaminoglycans. The conditions used for the introduction of functional groups are sometimes also causing reduction of the molecular  
30 weight of the polysaccharide.

Thus, not only it is possible to perform both steps by using UVC radiation, but it is possible to perform a first depolymerization step by using UVC radiation followed by a

second step using chemical-enzymatic depolymerization, or to perform a first step of chemical-enzymatic depolymerization followed by UVC radiation depolymerization.

The process of the present invention allows reduction of the molecular weight of the glycosaminoglycane without sensible modification of the chemical structure of the polysaccharide. The Mw of the glycosaminoglycane after irradiation is equal to or lower than 50% of the Mw of the glycosaminoglycane prior to irradiation

Absorption by a molecule of radiation in the ultraviolet (200-400 nm) or visible (400-800 nm) region of the spectrum can result in an excited state so high in energy that the energy absorbed is comparable in magnitude with the bond dissociation energies associated with organic molecules. If absorption occurs at 250 nm, the energy associated with this transition ( $E=480 \text{ kJ mol}^{-1}$ ) is greater than the bond dissociation energies of a carbon-carbon  $\sigma$ -bond ( $D\approx 347 \text{ kJ mol}^{-1}$ ), a carbon-oxygen  $\sigma$ -bond ( $D\approx 330 \text{ kJ mol}^{-1}$ ) and a carbon-hydrogen bond ( $D\approx 414 \text{ kJ mol}^{-1}$ ), average values in polyatomic molecules. It is not surprising therefore that chemical reaction can be induced by excitation with ultraviolet light. Generally speaking, homolytic cleavages of a covalent bond in organic molecules can be induced by UV radiation, forming two intermediate radicals.

Mercury vapour arc lamps are now the source of choice for most photochemical reactions in solution phase. These sources provide radiation in the ultraviolet and visible parts of the spectrum covering a range from 200 nm to 750 nm and so are useful for practically all purposes. There are three main type of mercury lamps designed as low, medium and high pressure and each has a different characteristic. The low pressure lamp operates at room temperature and mainly emits at 253.27 and 184.9 nm. Medium pressure lamps exhibit a weak continuum with a superimposition of spectral lines, associated with a diminished intensity at 253.27 and 184.9 nm. For the high pressure lamp, the large increase in pressure introduces many more lines. The emission below 280 nm is very weak. 189.4 nm radiation (far UV) is absorbed by the solvent, water. When absorption occurs at 184.9 nm, the energy associated with this transition is  $E=649 \text{ kJ mol}^{-1}$ . As the bond dissociation energy of oxygen-hydrogen is  $\approx 455 \text{ kJ mol}^{-1}$ , average value in polyatomic molecules, this radiation can generate  $\text{H}^\cdot$  and  $\text{OH}^\cdot$  from

water. 194.2 nm (far UV) and 253.27 nm are absorbed by the heparin molecule. Above 300 nm, heparin does not absorb.

The present invention relates to the use of UV radiation having an emission peak in the range from 245 nm to 260 nm, preferably from mercury lamps, most preferably low and medium pressure lamps.

In another embodiment the present invention is directed to the use of UV radiation prevalently at the wavelength of about 253 nm. In fact, it is possible to use filters that absorb the radiation at different wavelength and let pass only the 253 nm radiation. In this way it is possible to minimize side reactions as described before and obtain a high selectivity in the depolymerization reaction.

Without limiting the scope of the invention, it is believed that the radiation at 253 nm can produce dissociation of glycosidic bond and this is believed to be an important step in the depolymerisation. The excited molecule releases energy by forming two intermediate radicals, that somehow evolve to stable structures of lower  $M_w$ .

Although the mechanism of depolymerization is still unclear, we suppose that the formed free-radicals can be trapped by the oxygen present in water, and the resulting oxygen-oxygen bond can be cleaved with a mechanism similar to common autooxidation induced by atmospheric oxygen and sunlight (oxygen-oxygen  $\sigma$ -bond:  $D \approx 137 \text{ kJ mol}^{-1}$ ). We can describe this pathway to depolymerization,  $\text{C-O-C} \rightarrow \text{C-OH} + \text{C-OH}$ , as autooxidation of glycosidic bonds.

However, the process can be performed also in the absence of oxygen, indicating the presence of other mechanisms of evolution of the free-radicals which do not require reaction with oxygen.

The process of the invention is usually performed on a aqueous solution of the glycosaminoglycane.

The concentration of glycosaminoglycane in the solution can vary in a broad range. Preferably it is comprised between 2 and 25% w/v, more preferably between 4 and 15%. The pH of the aqueous solution is preferably kept in the range of 3.0 to 7.0, most preferably between 4.0 and 6.0. In fact, when the solution is basic, anionic depolymerization of the glycosaminoglycane takes place.

To regulate the pH to the desired value, it is possible to use weak acids such as acetic acid, citric acid and the like.

After irradiation, the solution is very clear and does not require any treatment to remove colored substances.

It is also possible to fractionate the intermediate depolymerized glycosaminoglycane by Gel Permeation Chromatography. The fractions containing the desired molecular weights are collected, concentrated by ultra filtration and lyophilized.

The process of the present invention is preferably performed by using a dynamic irradiation process.

With the term "dynamic irradiation process" it is meant a process wherein the solution to be irradiated is circulating as a thin layer in a lamp jacket and then returns to a reservoir where it is preferably thermostated. The liquid can circulate in one or more lamps which can be connected in series or in parallel.

The flow-rate of the solution in the circuit is not critical, but it is preferred to have a flow-rate high enough to avoid overheating of the solution.

The temperature of the solution in the circuit can vary in a broad range. Preferably it is maintained between 0 and 70 °C, more preferably between 10 and 60°C.

The process can be performed either in batch or in continuous mode. The apparatus is preferably formed of a reservoir from which the liquid moves to the irradiation area. The liquid is then returned to the reservoir.

In another embodiment of the invention, the solution is continuously withdrawn from the reservoir and subjected to membrane filtration with a cut off that can vary according to the desired target of MW.

For example, when willing to obtain depolymerised heparin with a  $M_w$  comprised between 2,000 and 3,000, it is possible to use a cut off of 5,000 Da. If the desired  $M_w$  is below 2,000 Da, then it is possible to use a cut off of 3,000 Da.

By the use of this continuous membrane filtration step, the solution which undergoes irradiation is maintained at a higher  $M_w$ . It is therefore possible to avoid formation of very small heparin fragments which present lower pharmaceutical activity.

#### Experimental section

Molecular mass ( $M_w$ ) was determined by size exclusion chromatography (European Pharmacopoeia 4<sup>th</sup> ed.: 2.2.30 and 2.2.46 for chromatography techniques and 01/2002:0828 p.1297 for method).

Absorbance at 260 nm was determined according to European Pharmacopoeia 4<sup>th</sup> ed 01/2002:0828 p.1297.

- Anti Xa activity was determined according to the method described in European Pharmacopoeia 4<sup>th</sup> ed.: 2.2.30 and 2.2.46 for chromatography techniques and  
5 01/2002:0828 p.1297 for method.

Anti coagulant activity was determined according to the method described in European Pharmacopoeia 4<sup>th</sup> ed.: 2.7.5 pg 168.

### Example 1

- 10 5 l of a 10% solution of heparin Na salt having Mw 13.000 Da, are introduced into the irradiation system formed of a reservoir, a circuit of 4 lamps (total W460), a peristaltic pump circulating the liquid into the circuit (19 l/h), and a small heat exchanger refrigerating the solution to 30 °C.

The liquid is irradiated for 16 hours.

- 15 Heparin is collected, spray-dried and analysed.

Mw = 5.000

Absorbance at 260 nm (solution 0.4%) = 0.080

Inorganic sulphates = absent

aXa activity = 106 U/mg

- 20 Anticoagulant activity = 114 U/mg

NMR: values obtained by intregation of <sup>13</sup>C-NMR signals

Sample	A <sub>NS6X-G</sub>	A <sub>NS6X-I<sub>2</sub>X</sub>	A <sub>NAC</sub>	A <sub>N,3,6S</sub>	A <sub>6S</sub>	I <sub>2S</sub>	I <sub>2OH</sub>	G
Starting heparin	12.92	68.08	13.6	4.6	75.9	61.4	13.26	25.7
Depolym. heparin	13.6	64.18	15.8	6.4	77.9	60.8	17.2	22

- The obtained intermediate depolymerised heparin can be fractionated by Gel Permeation Chromatography. The fractions containing the desired molecular weights  
25 are collected, concentrated by ultra filtration and lyophilized.

**Example 2**

Example 1 was repeated but continuously filtering the liquid with a membrane having a cut off value of 5.000 Da. The liquid removed by membrane filtration is continuously integrated by addition of starting 10% heparin-Na salt solution.

5

**Example 3**

Example 1 was repeated but using a 5 % dermatan sulphate (Mw = 35.000 Da) solution instead of a 10% heparin solution.

After irradiation, the recovered dermatan sulphate had the following characteristics:

10 Mw = 15.000 Da

Abs 260 nm = 0.150

Inorganic sulphates = absent

The obtained intermediate depolymerised dermatan sulphate can be fractionated by Gel Permeation Chromatography. The fractions containing the desired molecular weights are collected, concentrated by ultra filtration and lyophilized.

15

**Example 4**

2.5 l of a 10% solution of heparin Na salt having Mw 14.238 Da, were introduced into the irradiation system formed of a reservoir, a circuit containing a 115 W lamp, a peristaltic pump circulating the liquid into the circuit (25 l/h), and a small heat exchanger refrigerating the solution to 30 °C.

20

The solution was irradiated for 36 h and samples were taken to measure the Mw.

**Example 5**

25 Example 4 was repeated at 56 °C.

Table 1 reports the results obtained at 30 and 56 °C.

The results show that the increase of temperature from 30 to 56°C results in a higher depolymerization rate and a slight increase in the absorption at 260 and 280 nm, which remains however very low.

30

<b>Time (hours)</b>	<b>Example 4</b>	<b>Example 5</b>
	<b>30°C</b>	<b>56°C</b>
0	14238	14238
6	12718	11418
12	11975	10574
18		9930
24	10629	9000
36	9741	7829

<b>Analysis</b>	<b>Example 4</b>	<b>Example 5</b>
Abs at 260 nm	0,044	0,074
Abs at 280 nm	0,039	0,068

### Claims

1. Process for the depolymerization of glycosaminoglycanes to obtain depolymerised glycosaminoglycanes having a  $M_w$  equal to or lower than 50% of the original  $M_w$  prior to depolymerization, characterized by the use of UV radiation having a peak in the range from 245 nm to 260 nm
2. Process according to claim 1 wherein the source of UV radiation is a medium or low pressure Hg lamp.
3. Process according to claims 1-2 wherein the UV radiation has a prevalent emission band at 253 nm.
4. Process according to claims 1-3 wherein the glycosaminoglycane is heparin.
5. Process according to claims 1-3 wherein the glycosaminoglycane is dermatansulphate.
6. Process according to claims 1-5 wherein the solution subjected to depolymerization is kept at a temperature comprised between 0 and 70 °C.
7. Depolymerized glycosaminoglycane obtainable according to the process of claims 1-6

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP2004/050723

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 C08B37/00 C08B37/10

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data, PAJ, INSPEC, BIOSIS, COMPENDEX, IBM-TDB

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE CHEMICAL ABSTRACTS Chemical Abstracts Service, Columbus OH/USA; AN 136:134987 CA 2000, XP002255493	1,2,4,6, 7
Y	abstract	3
X	& KR 2000 012 173 A (REPUBLIC OF KOREA KONGKAE TAEHO KONGBO) 6 March 2000 (2000-03-06)	1,2,4,6, 7
Y	claims 1,2; example 15	3,5
X	E. A. BALAZS ET AL.: "Irradiation of Mucopolysaccharides with Ultraviolet Light and Electrons" RADIATION RESEARCH, vol. 11, 1959, pages 149-164, XP009018283 page 150, paragraph 4 page 162, paragraphs 1,2	1-3,6,7
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

5 August 2004

Date of mailing of the international search report

19/08/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Radke, M

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP2004/050723

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KURSHID A. KHAN ET AL.: "Chemical Effects of UV Radiation on Aqueous Solutions of Heparin-Ca Salt" POLYMER PHOTOCHEMISTRY, vol. 6, no. 6, 1985, pages 465-474, XP009018431 England cited in the application *The abstract* page 470, lines 8-14 -----	3
Y	WO 90/04607 A (OPOCRIN SPA) 3 May 1990 (1990-05-03) claims 1,5,6,10,11 -----	5
A	A. BLAZKOVA ET AL.: "Photocatalytic degradation of heparin over titanium dioxide" JOURNAL OF MATERIALS SCIENCE, vol. 30, no. 3, 1995, pages 729-733, XP009018286 page 730, left-hand column, paragraph 2 page 731, left-hand column, paragraph 3 -----	1-7

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/EP2004/050723

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
KR 2000012173	A	06-03-2000	NONE	
WO 9004607	A	03-05-1990	IT 1230582 B	28-10-1991
			AT 119547 T	15-03-1995
			AU 639427 B2	29-07-1993
			AU 4801290 A	14-05-1990
			DE 68921633 D1	13-04-1995
			DE 68921633 T2	19-10-1995
			WO 9004607 A2	03-05-1990
			EP 0439555 A1	07-08-1991
			ES 2021905 A6	16-11-1991
			JP 2960158 B2	06-10-1999
			JP 4502928 T	28-05-1992
			KR 148799 B1	17-08-1998